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Evaluation of protective effect of L-carnitine and N-acetylcysteine in mesenteric ischemia-reperfusion injury model in rats

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Abstract
Aim: Ischemia-reperfusion injury is an important dilemma in surgical modalities. The mechanism of IR damage is related to oxidative stress mediators. L-carnitine and N-acetylcysteine are thought to have antioxidant activity. In our study, we aimed to evaluate the effects of short-term administration of these two drugs and to compare their effects on oxidative stress parameters in the experimental mesenteric ischemia-reperfusion model in rats.
Materials and Methods: Twenty-four female Sprague-Dawley rats were allocated into 3 experimental groups. In Group 1 (CG) (n=8), rats underwent occlusion of the superior mesenteric artery for 30 minutes and were not given any medications. In Group 2 (NG) and Group 3 (LG), rats underwent occlusion as CG. Rats were given 150 mg/kg (IP) N-acetylcysteine and 300 mg/kg IP L-carnitine according to their groups 15 minutes before reperfusion. Rats were sacrificed with high dose anesthetic drugs after 60 minutes of reperfusion. Blood and liver tissue samples were obtained to investigate total oxidant status (TOS), total antioxidant status (TAS), and oxidative stress index (OSI).
Results: By biochemical examination, all groups showed similar levels of TOS. There was no statistically significant difference between the level of TAS and OSI in all groups (p>0,05). There was no any statistically significant difference between the groups for TAS measurements (p=0.061; p>0.05); however, higher measurement values in the L-Carnitine group is considerable.
Discussion: Although the use of drugs with proven antioxidant efficiency after ischemia may cause a histologically significant difference in IR injury, there was no significant efficiency in the reduction of superoxides in the circulation. Therefore, we believe that the use of NAC and L-carnitine as antioxidants after the development of ischemia does not help to prevent intestinal IR injury.

Keywords
Ischemia, L-carnitine, Mesenteric, N-acetylcysteine

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Introduction

Mesenteric ischemia is a vascular emergency condition with a high potential for fatal progression [1]; therefore, early diagnosis and treatment are essential. Although diagnostic methods have recently improved, the diagnosis of this condition depends on clinical suspicion [2]. It is obvious that the primary objective after diagnosis is to prevent ischemia-dependent necrosis which may be achieved only with reperfusion.

Intestinal ischemia-reperfusion (IR) is one of the significant surgical dilemmas for patients with mesenteric ischemia [3]. Reperfusion damage appears in the intestinal area by the achievement of the blood supply into the occluded artery. Although the mechanism of damage generation has not yet been clarified, the generally accepted hypothesis is the important role of oxidative stress mediators [3]. Besides, an increase of the capillary permeability, accompanied by vasodilatation after intestinal reperfusion, causes leakage of fluid and erythrocytes into the intestinal lumen; this leads to capillary plugs. Such plugs cause a decrease in the blood supply of the tissue again, which is called the "No-reflow phenomenon" [4]. Due to oxidative stress mediators and "No-reflow phenomenon", no blood supply into the tissue can be achieved, and the tissue cannot revert even if the occlusion is eliminated. Like the disease itself, reperfusion damage also appears with high mortality and morbidity.

Carnitine is an endogenous ester with well-defined metabolic effects in the literature which transports long-chain fatty acids into the mitochondria for beta-oxidation [5]. It is known that carnitine acts as a cleaner for free oxygen radicals, suppresses the oxidative stress, and reduce lipid peroxidation [6]. It was considered to play a protective role in metabolic diseases for a long period. Furthermore, it is known for many years that carnitine reduces ATP loss and prevents acidosis. It reduces the damage through anti-radical efficiency and acts as an energy source in reperfusion damage. Carnitine stabilizes the cytosolic membrane and prevents the release of cytotoxic enzyme. The protective effect of carnitine on endothelium is also well-known. Such effects play an important role in the reduction of the "No-reflow phenomenon" [3].

N-acetylcysteine (NAC) is a small molecule including a thiol group which has been popularly analyzed recent years [7]. NAC is known to be effective in the reduction of endothelial dysfunction, inflammation, and fibrosis [8]. The thiol group involved in the molecule gives the hydrogen ion to oxygen radicals easily, reduces the radicals into the water; and thereby mitigates the epithelial injury induced by H2O2. The aforementioned reaction is caused by the direct antioxidant effect of NAC whereas it creates an indirect antioxidant effect through glutathione [9].

The present study aimed to evaluate the protective effect of the aforementioned molecules, which were analyzed in different aspects in the literature, on a mesenteric ischemia-reperfusion model created experimentally on rats and to compare them with each other.

Material and Methods

Animals and Experimental Design

The present experimental study was performed after approval of the Ethical Committee of Animal Experiments within Mehmet Akif Ersoy Experimental Research and Development Training Center in Istanbul. All experimental and surgical procedures in animal experiments were performed at the Mehmet Akif Ersoy Experimental Research and Development Training Center in Istanbul. National guidelines and regulations for the care and use of animals have been followed.

In the present study, 24 8-month-old Sprague-Dawley female rats with bodyweight between 250 g and 350 g living under standard environment conditions (22°C, individual steel cage, 12-hour day and 12-hour night cycle, ad libitum feeding) were used. All rats were operated after a fasting period of 12 hours and they were allowed to drink water within those 12 hours before the procedure. The rats were randomly divided into 3 groups including 8 rats in each group as follows: Group 1 (Control group = CG); Group 2 (N-acetylcysteine Group = NG); Group 3 (L-carnitine Group = LG).

Surgical Procedures

The animals included in the experiment were sedated with intraperitoneal xylazine hydrochloride (5 mg/kg; Rompun® 2%, Bayer) and anesthetized with intraperitoneal ketamine hydrochloride (35 mg/kg; Ketalar®, Pfizer) before the surgical procedure. Spontaneous ventilation of the animals was enabled during the experiment period. The rats were taken onto the operating table on a warming pad (12 DVC voltage- 2,5 A-Kent Scientific Corporation) in an operating room environment at 14.8°C temperature and 42% humidity; a 3cm laparotomic incision was performed on the midline after dermal sterilization through Betadine® and the intestinal tissue deviated to the left. The superior mesenteric artery was dissected by a forceps and flow was measured through laser doppler (Periflux System 5000, Perimed) before mesenteric ischemia. The superior mesenteric artery was sutured to interrupt the flow-through 6-0 silk in a re-openable pattern after the measurement. Ischemia was confirmed through laser doppler again in all groups after the ischemia; and flow measurements were done (Figure 1). No drug or placebo was administrated to the control group during and after ischemia; 150 mg/kg intraperitoneal N-acetylcysteine was administered to Group 2 and 300 mg/kg L-carnitine was applied to Group 3 at 15th minute of ischemia. Intraperitoneal administration was used to supply a large number of drugs in the animal system.

The silk suture (6-0) was opened at 30th minute after ischemia and reperfusion were achieved. The achievement of flow was confirmed by laser Doppler. An intracardiac blood sample of 5 ml was obtained from each rat at the 90th minute of the study while the rats were still under anesthesia. All rats used in the study were sacrificed through a high dose of intraperitoneal ketamine hydrochloride (45 mg/kg; Ketalar®, Pfizer).

Biochemical Analysis

Blood samples were collected by cardiac puncture from the participants at the end of the 12-hour fasting period. Blood samples were collected into dry tubes and centrifuged at 3500 × g at 4 °C for 10 min. The serum was collected and stored at − 80 °C until further analysis.

Total Antioxidant Status (TAS) determination

The total antioxidant capacity of plasma was measured using an automated colorimetric measurement method developed by Erel [10]. Total Antioxidant Status (TAS) levels were measured

spectrophotometrically by a commercial kit (Rel Assay, Gaziantep, Turkey). Antioxidants in the sample reduce dark blue-green colored ABTS radical to colorless form. The change of absorbance at 660 nm is related to the total antioxidant level of the sample. The assay is calibrated with a stable antioxidant standard solution which is traditionally called a Trolox Equivalent that is vitamin E analog. Total antioxidant activities of the samples were expressed in mmol Trolox Equiv/L.

Total Oxidant Status (TOS) determination

The total oxidant capacity of plasma was measured using an automated colorimetric measurement method developed by Erel [11]. Total Oxidant Status (TOS) levels were measured by a spectrophotometric method by a commercial kit (Rel Assay, Gaziantep, Turkey). Oxidants present in the sample oxidize the ferrous ion chelator complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are abundantly present in the reaction medium. The ferric ion makes a color complex with chromogen in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μm H202Equiv/L).

The ratio of TOS to TAS was accepted as an oxidative stress index (OSI). For the calculation; the unit of TAS was converted to mmol/L, and the OSI value was calculated according to the following formula: OSI (arbitrary unit): TOS (μmH202Equiv/L) / TAS (mmolTroloxEquiv/L) [12].

Statistical Analyses

NCSS (NumberCruncher Statistical System) 2007 (Kaysville, Utah, USA) program was used for statistical analyses. Descriptive statistical methods (first quartile, third quartile, and median) were used to assess the study data. The conformity of quantitative data to normal distribution was tested by the Shapiro-Wilk test and graphical reviews. The Kruskal-Wallis test was used to compare three and more groups without normal distribution. The significance was assessed at $p<0.05$ level.

Results

The first quartile, third quartile, and median values of TAS in the blood were 2.282, 3.183, and 2.594, respectively whereas the first quartile, third quartile, and median values of TOS were 57.008, 97.598 and 77.593, respectively. The first quartile, third quartile, and median values of OSI were 1.855, 3.663, and 2.804, respectively (Table 1).

There was no statistically significant difference between the groups in terms of TAS, TOS, and OSI measurements ($p>0.05$) (Table 2).

For TAS analyzed in the liver tissue; the first quartile, third quartile, and median values were 0.386, 0.645, and 0.504. The first quartile, third quartile, and median values of TOS were 19.310, 31.172, and 23.952. The first quartile, third quartile, and median values of OSI were 4.376, 5.728, and 4.878.

There was no statistically significant difference between the groups in terms of TAS measurements ($p=0.061$; $p>0.05$); however, higher measurement values in the L-Carnitine group is considerable. (Table 3)

Table 1. Distribution of TAS, TOS, and OSI Measurements

	Q1-Q3	Median
TAS (mmol/L)	2.282-3.183	2.594
TOS (μmol/L)	57.008-97.598	77.953
OSI (AU)	1.855-3.663	2.804

Q1:First quartile, Q3:Third quartile, AU(Arbitrary Unit) = TOS (μmol H2O2 Eq/L) / TAS (μmol Trolox Eq/L) x 10

Table 2. Evaluation of TAS, TOS, and OSI Measurements According to the Groups

		Control group (n=8)	N-acetylcysteine group (n=8)	L-carnitine group (n=8)	p
TAS (mmol/L)	Q1-Q3	2.272-3.402	2.144-2.999	2.581-3.427	0.275
	Median	2.871	2.318	2.633	
TOS (μmol/L)	Q1-Q3	54.528-98.898	59.094-104.449	48.937-96.535	0.996
	Median	78.425	75.433	83.701	
OSI (AU)	Q1-Q3	1.837-3.655	2.508-4.538	1.748-4.162	0.533
	Median	2.203	3.347	2.393	

Q1:First quartile, Q3:Third quartile, AU(Arbitrary Unit) = TOS (μmol H2O2 Eq/L) / TAS (μmol Trolox Eq/L) x 10, Kruskal Wallis Test

Table 3. Evaluation of TAS, TOS, and OSI Measurements in Liver Tissue

		Control group (n=8)	N-acetylcysteine group (n=8)	L-carnitine group (n=8)	p
TAS (mmol/L)	Q1-Q3	0.352-0.520	0.292-0.636	0.483-0.762	0.061
	Median	0.404	0.498	0.615	
TOS (μmol/L)	Q1-Q3	19.159-29.405	17.964-31.813	24.752-32.070	0.196
	Median	23.225	19.811	29.310	
OSI (AU)	Q1-Q3	4.721-5.728	4.045-6.484	3.953-5.472	0.471
	Median	5.184	4.963	4.693	

Q1:First quartile, Q3:Third quartile, AU(Arbitrary Unit) = TOS (μmol H2O2 Eq/L) / TAS (μmol Trolox Eq/L) x 10, Kruskal Wallis Test

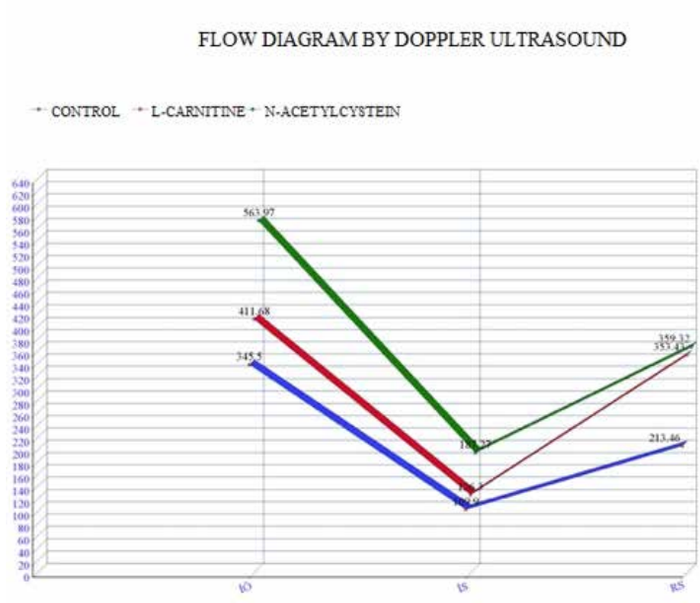

Figure 1. Laser Doppler measurements in the rats of which ischemia model was created before and after ischemia as well as after reperfusion (Unit).

Discussion

IR damage is a common phenomenon in surgical pathologies [13]. The pathophysiological events caused by IR are likely to be observed more commonly in the intestinal, cardiac, and cerebral tissues [14]. Reperfusion in the ischemic tissues causes a series of events in the affected tissue. The lipids located on the membranes of the cell organelle, which plays an important role in this cascade, are vulnerable against injury by free radicals, and lipid peroxidation appears when such free radicals react with the lipids. Such reactions create many toxic derivatives, such as superoxide anion, hydrogen peroxide, peroxynitrite, and hydroxyl radicals which would disrupt the cellular function [15, 16].

One of the important mechanisms in the pathogenesis of IR damage is the inflammatory pathways [15]. Inflammatory cytokines and neutrophil infiltration were known to be effective in the injury. Due to the aforementioned mechanisms, treatment regimens should be designed to prevent tissue injury and irreversible damage that was defined before. Possible anti-inflammatory, anti-apoptotic and anti-oxidant agents that may be effective for treatment were analyzed in IR injury that appeared in different organs. The efficiency of some agents such as sodium nitroprusside, atorvastatin, MgSO4, mannitol, ethyl private was tested in different IR injuries [7, 13, 15, 17, 18]. We reviewed the efficiency and superiority of L-carnitine and N-acetylcysteine on the IR injury model created on the animal model.

It is well known that L-carnitine transports long-chain fatty acids for beta-oxidation and may scavenge free oxygen radicals and reduce lipid peroxidation [5]. Therefore, it has been subject to many metabolic studies. Virmani et al. examined the neuroprotective effects of L-carnitine and stated that L-carnitine enables mitochondrial transmission, regulates the activity of permeability transmission pores and associatively reduce the peroxynitrite level [19]. Although this is theoretically possible, we did not detect any statistically significant difference between the L-carnitine group and the control group in terms of TAS, TOS, and OSI values. This may be explained by the different characteristics of the analyzed tissues and the difference in energy metabolisms used in the tissues.

Hosgorler et al. examined tissue and blood samples after reperfusion for 3 hours following L-carnitine administration at 8th minute of ischemia for 10 minutes and detected that morphological injury is statistically less, whereas perfused microvessels and epithelial regeneration were statistically higher in the rats treated with L-carnitine [3]. Such difference may be explained by longer ischemia period and shorter reperfusion period in the present study when compared with the study conducted by Hosgorler et al. Because the damage that appeared in intestinal ischemia is known to be dependent on ischemia duration and intensity [3].

Kalimeris et al. did not detect any difference between oxidative indicators in the plasma in their experimental study where IR injury was created to compare NAC doses [20]. In the studies where the protective effect of the agents such as NAC and atorvastatin was significant, the aforementioned drugs were administrated to the animals before the creation of ischemia [15,20, 21]. Another study focusing on the protective effect of NAC and atorvastatin in IR damage created in the colon did not detect any significant difference in superoxide dismutase activity [22]. A previous study analyzing the IR injury on renal tissue did not detect any statistically significant difference in superoxide dismutase levels between non-treated IR group and NAC- and erdosteine-treated rats. There is a significant difference in renal tissue histology of the group treated with erdosteine which started to be administrated 2 days before the creation of ischemia [23]. Baumann et al. performed an experimental study with hepatic ischemia and reported that short-term use of NAC is not successful for the preservation of hepatic functions [24]. A review on IR injury in an experimental model stated positive efficiency on the markers associated with liver damage of NAC which was used before the formation of ischemia [25]. Lack of a significant difference in the levels of oxidative biomarker of NAC and L-carnitine, which we administer after the creation of ischemia, is consistent with some studies in the literature.

Limitations

We declare that our study has limitations. These limitations include the small number of animals per group, the potentially unknown effects of the presence of the anesthetic agents on the parameters measured, the absence of histopathological evaluation of intestines, and whether the agents intraperitoneally administered were actually absorbed by the bowel.

Conclusion

Although the use of the drugs with proven antioxidant efficiency after ischemia may cause a histologically significant difference in IR injury, no significant efficiency was shown in the reduction of superoxides in the circulation. Therefore, we believe that the use of NAC and L-carnitine as antioxidants after the development of ischemia is not useful to prevent intestinal IR injury in the emergency medicine departments.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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